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Severe Mental Retardation and Macroorchidism Without Mutation in the FMR1 Gene

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Only one missense mutation, an Ile304Asn, has been reported in the fragile X gene (FMR1). This mutation is located in the second KH domain of FMR1, and has led to the discovery of the function of the FMR1 gene product as an RNA-binding protein. The patient carrying this mutation has profound mental retardation, macroorchidism, and an "acromegalic" face with prominent supraorbital ridges, enlarged jaw, heavy brow ridges, thick lips, and a broad nose. We have studied the possible involvement of FMR1 in two maternal half-brothers with a phenotype similar to that of the patient with the Ile304Asn mutation. Both brothers had an identical number of CGG repeats in the normal size-range, and shared the same maternal Xq27 haplotype. Southern blot analysis with two overlapping FMR1 cDNA clones, spanning the total FMR1 open reading frame, showed no major deletions, insertions, or gross rearrangements. Single-strand conformation pattern (SSCP) analysis of the KH domains showed no aberrant patterns. The total open reading frame of the FMR1 gene was cloned and sequenced, but no mutation was found. Northern blot analysis showed mRNA in the normal size-range, and immunocytochemistry on individual lymphocytes indicated that FMRP, the protein product of FMR1, was present. In conclusion, it is unlikely that FMR1 plays a role in the phenotype of this patient. Other genes may be responsible for the combination of mental retardation and macroorchidism.

KEY WORDS: fragile X syndrome, macroorchidism, FMR1 gene, RNA binding, KH domain, X-linked mental retardation

INTRODUCTION

Lack of expression of the FMR1 gene results in fragile X syndrome [Willems, 1994]. In most cases, this is caused by elongation of an untranslated CGG repeat above a threshold number of repeats, which downregulates transcription of the gene [Verkerk et al., 1991; Pieretti et al., 1991; Verheij et al., 1993]. In a few patients, deletions involving all or part of the FMR1 gene, or a single nucleotide deletion or a two base-pair substitution, are responsible for the absence of FMR1 expression [reviewed in Kooy et al., 1996]. A missense mutation was reported in only one patient [De Boulle et al., 1993]. That patient had unusually severe mental retardation and macroorchidism, an "acromegalic" face with prominent supraorbital ridges, broad nose, thick lips, and heavy eyebrows, spastic paraparesis, muscle atrophy, and hypotrophy of the lower limbs. An Ile304Asn missense mutation resulting in the presence of a mutant FMR1 protein was found in this patient. This mutated FMR1 protein is responsible for a more severe phenotype than reduction of the amount of protein by CGG amplification or deletion. This suggests a possible gain-of-function of the mutant protein [Willems, 1994]. The Ile304Asn missense mutation is located in an RNA-binding KH domain of FMR1 [Ashley et al., 1993; Siomi et al., 1993], and reduces the RNA-binding capacities of the FMR1 protein (FMRP) in vitro [Siomi et al., 1994; Verheij et al., 1995]. After we found this first intragenic FMR1 mutation in 1993, we anticipated that additional intragenic mutations would be found. However, up to now, only two other intragenic FMR1 mutations, both leading to the absence of FMRP, have been described [Lugenbeel et al., 1995].

Tariverdian et al. [1991] described a family with a clinical presentation comparable to that of the patient described by De Boulle et al. [1993]. Two maternal half-brothers in this family have mental retardation, macro-

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TABLE I. Comparison of Clinical Findings

Finding	Tariverdian et al. [1991] I	Tariverdian et al. [1991] II	De Boulle et al. [1993]	Fryns et al. [1986] I	Fryns et al. [1986] II	Fryns et al. [1986] III	FRA(X)	Atkin et al. [1985]	Clark and Baraitser [1987]
Mental retardation	+	+	+	±	+	+	+	+	+
Macroorchidism	+	±	+	+	+	+	+	+	+
Acromegalic face	+	+	+	+	+	+	−	−	−
Facial asymmetry	+	+	+	+	−	−	−	−	−
Large skull[a]	+	+	+	+	+	−	+	+	+
Long face	+	+	+	+	+	+	+	−	−
Enlarged jaw	+	+	+	+	+	+	+	−	−
Thick lips	+	+	+	+	−	−	−	+	+
Heavy eyebrows	+	+	+	+	−	+	−	−	−
Broad nose	+	+	+	+	+	+	−	+	+
Aggressiveness	+	+	+	+	−	−	−	−	+

[a] OFC >57 cm.

orchidism, and an acromegalic face, as did the patient of De Boulle et al. [1993] (Table I). The mother of both half-brothers has a similar, albeit less severe, clinical picture than her sons, including facial anomalies and mild mental retardation. This suggests X-linked inheritance.

To verify whether FMR1 mutations are responsible for this phenotype, we analyzed the FMR1 gene of one of the half-brothers in detail.

MATERIALS AND METHODS

Linkage Analysis

The intragenic CGG repeat, FMRa polymorphism [Kunst and Warren, 1994], and microsatellite marker FRAXAC2, the microsatellite markers DXS292 (VK14), DXS297 (VK23), DXS548 (RS46), FRAXAC1, DXS1491, and P39 [polymerase chain reaction (PCR) marker], and the VNTR marker DXS52 (F814) were used to investigate whether patients 1 and 2 had inherited the same maternal Xq27–28 chromosomal region. PCR analysis of the CGG repeat was performed as described previously [Fu et al., 1991; Kooy et al., 1996]. Analysis of the intragenic marker FMRa was performed as described by Kunst and Warren [1994].

Southern Blot Analysis

The length of the CGG repeat in the FMR1 gene was studied by Southern blot analysis with the pP2 probe [Verkerk et al., 1991; Kooy et al., 1996]. Large deletions and other rearrangements in the FMR1 gene were studied using the overlapping FMR1 cDNA clones BC22 and BC72 [Verkerk et al., 1991] on *Msp*I, *Bgl*II, *Eco*RI, and *Taq*I digests of genomic DNA.

Detection of the Ile304Asn Mutation

The presence of the Ile304Asn mutation was studied as previously described [De Boulle et al., 1993].

SSCP Analysis of KH Domains

SSCP analysis of exons 8–10 containing the complete sequence of the KH domains was performed with genomic primers KHa (5′-AGTGTATTCATCAGACGTCCA-3′) and KHb (5′-GTACTATATGTCAAGCAGATAC-3′) for the first KH domain located in exon 8, and KHc (5′-ACCAAAACTTGATTTATTTATTTC-3′) and KHd (5′-ATATGTGCCACAAAATATTCG-3′), for the second KH domain located in exons 9–10. One hundred nanograms of genomic DNA were amplified in a standard PCR reaction with 35 cycles, an annealing temperature of 55°C, and ^{32}P-labeled dNTPs. PCR products were electrophorized on nondenaturing hydrolink MDE gels (JT Baker, Phillipsburg, NJ) at room temperature and at 4°C.

Northern Blot Analysis

Total RNA was extracted from EB-lymphoblastoid cells with TRIzol reagent (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's instructions. Twenty micrograms of total RNA were precipitated and resuspended in 12.5 μl deionized formamide, 2.5 μl 10 × MOPS, and 4 μl formaldehyde (37%), heated for 5 min at 65°C, and chilled on ice before 2.5 μl of dye marker (50% glycerol, 0.1 mg/ml bromophenol blue) were added. The samples were loaded on a 1% formaldehyde-agarose gel and electrophorized in 1 × MOPS buffer at 100 V for 3 hr. After electrophoresis, the gel was soaked in distilled water and in 10 × SSC for 15 min each, and blotted onto a Hybond N^+ nylon membrane (Amersham, Amersham, UK) in 10 × SSC for 12–16 hr. RNA was immobilized onto the membranes by baking for at least 2 hr at 80°C. The membranes were hybridized with a ^{32}P-α-dC/ATP-labeled cDNA clone BC22. Hybridization was performed in 6 × SSC, 10 × Denhardt's, 0.1% SDS, 10% dextran sulphate at 60°C for 16–20 hr. Filters were washed in 0.3 × SSC, 0.1% SDS at 65°C, followed by autoradiography on X-OMATK film (Kodak) in a cassette with intensifying screens for 48 hr.

Sequence Analysis

First-strand cDNA from total RNA was synthesized with the Superscript preamplification system (Gibco-BRL). Four pairs of primers K1–K8 (Fig. 2) were used to PCR-amplify the coding region of the FMR1 gene, as described previously [De Boulle et al., 1993]. Two microliters of first-strand cDNA were used as template. PCR reactions with primer sets K1–K2 and K3–K4 resulted in one RT-PCR product, visible as a single discrete band on ethidium bromide-stained agarose gels. This band was extracted from the agarose gel and subcloned into pBluescriptSK(−) (Stratagene, La Jolla,

CA). PCR reactions with primer set K5–K6 resulted in two RT-PCR products, and PCR reactions with primer set K7–K8 resulted in six RT-PCR products due to alternative splicing [Verkerk et al., 1993]. The two RT-PCR products of K5–K6 could be visualized as discrete bands on ethidium bromide-stained 3% agarose gels, but only 4 of 6 RT-PCR products of K7–K8 could be separated on a 3% agarose gel. The largest band of K5–K6, containing the alternatively spliced region I, and the largest RT-PCR fragment amplified with K7–K8, containing the alternatively spliced regions II–IV (Fig. 2), were extracted from the agarose gel and subcloned into pBluescriptSK(−). The subclones were manually sequenced with vector primers, using the Sequenase version 2.0 kit (USB, Cleveland, OH), and sequenced with a dye primer sequencing kit on the ABI 373A automated sequencer. Additionally, the bands of K7–K8 were separated on a 3% agarose gel, extracted from the agarose gel using a spin bind kit (FMC, Rockland, ME), and directly sequenced on the ABI automated sequencer using a cycle-sequencing reaction with dye terminators. The shorter products were sequenced to detect the nucleotides in the middle of the large K7–K8 product, as some remained ambiguous after sequencing the largest fragment. Primers K10 (5′-TGGTCTGTGTATATAACAACTA-3′) and K11 (5′-GGTATAGGAAATATAACTTCAG-3′) were used to amplify and sequence the genomic DNA sequence of the 3′ end of the open reading frame, as the K8 primer is located within the coding sequence.

FMR1 Protein Detection Test

The immunocytochemical test to visualize FMRP directly in cells fixed on slides [Willemsen et al., 1995] was performed as described in detail [Kooy et al., 1996], on cultured EB-lymphoblastoid cells of the patient, using FMR1 antibody 1a [Devys et al., 1993].

RESULTS

The patient is shown in Figure 1.

We first investigated whether the two half-brothers had inherited the same maternal FMR1 gene. Results

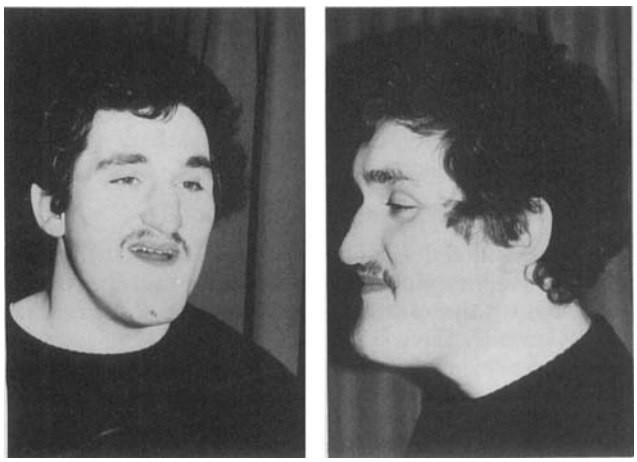

Fig. 1. Facial appearance of patient. Note acromegaloid appearance with facial asymmetry, long face, enlarged jaw, thick lips, heavy eyebrows, and broad nose.

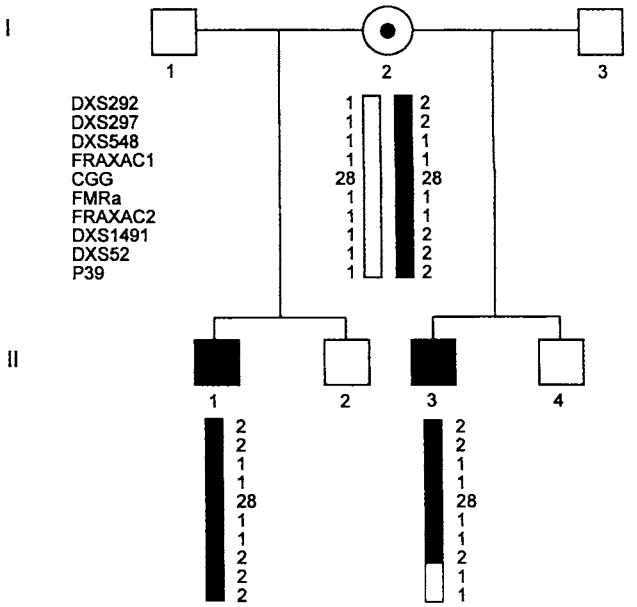

Fig. 2. Xq27–28 haplotypes of the 2 half-brothers and the mother. Both half-brothers share the same haplotype around FMR1 with a likelihood of >99%.

of the linkage analysis are shown in Figure 2. None of the intragenic markers used (CGG repeat, FMRa, and FRAXAC2) were informative in the mother (Fig. 2). The closest informative proximal marker was DXS297 at 3 cM from FMR1 [Willard et al., 1994], and the closest distal marker was DXS1491, located at 600 kb from FMR1 [Hamel et al., 1994]. The likelihood that both brothers share the same maternal FMR1 gene is thus >99%. Therefore, one of the brothers, patient 1 in Tariverdian et al. [1991], was selected for further analysis of the FMR1 gene.

The patient had 28 CGG repeats as determined by PCR analysis, which is within the normal size range. No methylation of the promotor region of FMR1 was apparent on Southern blots (data not shown). Deletions of part of the FMR1 gene or other gross gene rearrangements could not be found, when genomic DNA digested with *Msp*I, *Bgl*II, *Eco*RI, and *Taq*I was hybridized with clones BC72 and BC22, together spanning the entire FMR1 cDNA sequence (data not shown).

The Ile304Asn mutation previously described in a severely affected patient [De Boulle et al., 1993] was not present. As this missense mutation lies within one of the KH domains of FMR1, these domains were subjected to SSCP analysis of genomic PCR fragments. No abnormal patterns were identified (data not shown). Subsequently, the whole open reading frame of the FMR1 gene was sequenced. After RT-PCR, cDNA was sequenced using the four primers sets K1–K2, K3–K4, K5–K6, and K7–K8 (Fig. 3). The largest possible PCR products, including all possible splice variants, were sequenced, either directly or after subcloning. As K8 includes the last 21 base pairs of the coding sequence, a fifth primer set, K10–K11, was designed to amplify the

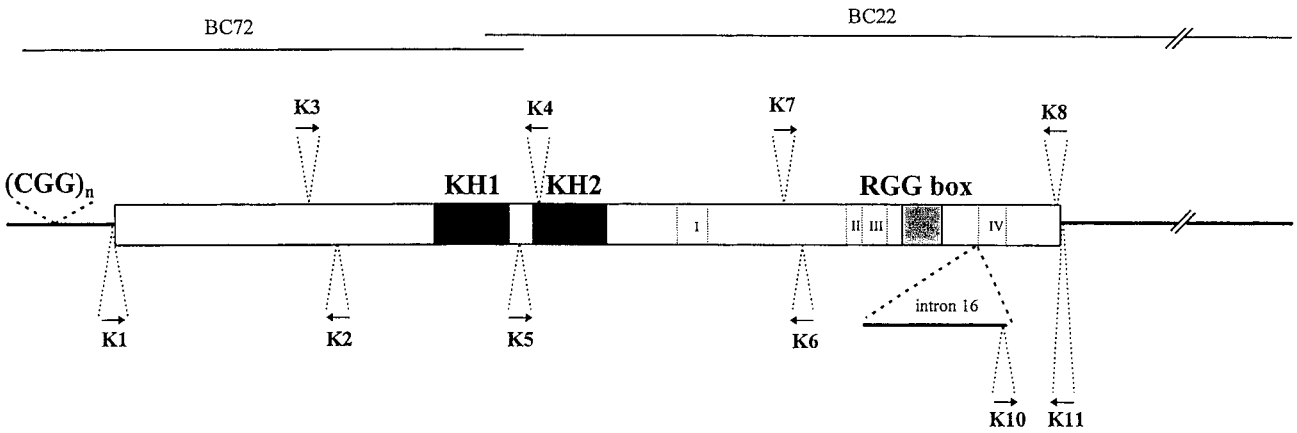

Fig. 3. Schematic representation of FMR1 gene. RNA-binding KH domains and RGG box are indicated in black and grey, respectively. Position of primers for sequence analysis and probes for Southern blot analysis are given. Roman numerals represent exonic regions not always included in the final transcript due to alternative splicing.

3′ region at the genomic level. The FMR1 sequence from the patient corresponded completely with the previously described sequence [Verkerk et al., 1993].

Northern blot analysis after hybridization with probe BC22 showed that the FMR1 mRNA from the patient was of the same length as normal FMR1 mRNA. The amount of mRNA seemed normal, although no densiometric analysis was performed (Fig. 4). FMR1 protein (FMRP) was detectable in >95% of individual lymphocytes after protein visualization with streptavidin-biotin-alkaline phosphatase.

DISCUSSION

Mental retardation and macroorchidism suggest the presence of fragile X syndrome. However, several patients with these findings but without cytogenetic (fragile site) and/or molecular evidence (CGG repeat amplification) of fragile X syndrome have been described. In these patients it was unclear whether their phenotype was due to other inherited or acquired factors, or to FMR1 mutations different from the classical CGG amplification. Evidence for the first hypothesis was put forward by Fryns et al. [1986], who identified such patients with acquired lesions of the central nervous system (especially the hypothalamic region). Evidence for the second hypothesis was found when we identified an Ile304Asn missense mutation in a patient with severe mental retardation and macroorchidism [De Boulle et al., 1993]. As the latter patient had a very similar phenotype to the patient reported by Tariverdian et al. [1991] (Table I), we studied the FMR1 gene in this family. It is unlikely that the patients in our family have a mutation in FMR1, as 1) the promotor region of FMR1 has a CGG repeat of normal size and is unmethylated, 2) FMR1 mRNA and FMRP are present, and 3) the sequence of the open reading frame of FMR1 is normal. The 5′ and 3′ untranslated regions of the gene have not been sequenced, but gross DNA rearrangements seem unlikely, as 1) Southern blot hybridization with cDNA probes spanning the total transcribed sequence did not reveal any abnormalities, and 2) the mRNA is of normal length on Northern blots. Base substitutions or small rearrangements in the promotor region may have gone undetected, but are unlikely as normal amounts of mRNA and FMRP were found. Altogether, it is unlikely that FMR1 is involved in the cause of the symptoms of this patient. This suggests that next to FMR1, there are other genes on the X chromosome responsible for the combination of mental retardation and macroorchidism, which is in line with the frequent observation of this combination of symptoms in fragile X-negative males [Volke et al., 1990]. Corpus Callosum Hypoplasia, Retardation, Adducted Thumbs, Spastic Paraparesis, and Hydrocephalus (CRASH) syndrome, a frequent cause of X-linked mental retardation [Fransen et al., 1995, 1996], is also unlikely, as linkage analysis excluded the L1 gene, located distally from DXS52 (Fig. 1).The 2 patients of our family share some similarities with patients with Clark-Baraitser syndrome [Clark and Baraitser, 1987; Baraitser et al., 1995] and with Atkin-Flaitz syndrome [Atkin et al., 1985]. Both syndromes are X-linked conditions with a combination of mental retardation, macroorchidism, macrocephaly, prominent supraorbital ridges, and prominent lower lip (Table I). Splitters and lumpers might have another semantic discussion as to whether or not our family might fit into the clinical spectrum of these or other conditions, but

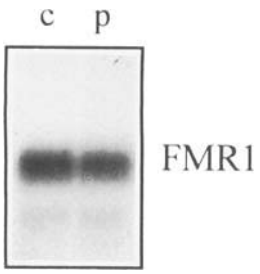

Fig. 4. Northern blot of total mRNA of our patient (p) compared with a control (c), hybridized with FMR1 cDNA probe BC22. A transcript of 4.8 kb is visible both in our patient and in the normal control.

the molecular geneticist will probably have the last word by identification of the respective gene defects.

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